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# Molecular Location of a Species-Specific Epitope on the Hamster Scrapie Agent Protein

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**Scrapie is a transmissible neurodegenerative disease of sheep and goats. An abnormal host protein, Sp33-37, is the major protein component of the scrapie agent and the only known disease- or agent-specific macromolecule. Two monoclonal antibodies (MAbs), 4H8 (immunoglobulin G2b [IgG2b]) and 6B11 (IgG1), produced by immunizing mice with the intact hamster 263K scrapie agent protein, Sp33-37$^{Ha}$, were found to have species specificity similar to that reported previously for MAb 3F4 (IgG2a), which was produced by using PrP-27-30 as the immunogen (R. J. Kascsak, R. Rubenstein, P. A. Merz, M. Tonna-DeMasi, R. Fersko, R. I. Carp, H. M. Wisniewski, and H. Diringer, J. Virol. 61:3688-3693, 1987). These antibodies all bound to Sp33-37 derived from hamster but not from mouse cells. Competitive binding assays demonstrated that all three MAbs bound to the same or overlapping sites on Sp33-37$^{Ha}$. The molecular location of the epitope for these antibodies was determined to within 10 residues by using an antigen competition enzyme-linked immunosorbent assay in which synthetic peptides spanning Sp33-37$^{Ha}$ residues 79 to 93 or 84 to 93 specifically inhibited binding of these antibodies to plates coated with purified Sp33-37$^{Ha}$. A synthetic peptide with the mouse-specific sequence (83 to 92) that differed from the hamster sequence by substitution at two positions ($Met_{Ha}$-87 → $Leu_{Mo}$-86 and $Met_{Ha}$-90 → $Val_{Mo}$-89) did not inhibit antibody binding to Sp33-37$^{Ha}$. MAb 3F4 binding to hamster Sp33-37 was eliminated by chemical modification of Sp33-37$^{Ha}$ with diethylpyrocarbonate or succinic anhydride and by cleavage with CNBr or trypsin. The effect of diethylpyrocarbonate on MAb 3F4 binding was not reversed by hydroxylamine treatment. MAb 3F4 binding was not affected by prolonged exposure of Sp33-37$^{Ha}$ to 70% formic acid or by boiling in sodium dodecyl sulfate. We conclude that the epitope for these MAbs is a linear determinant that includes Met-87, Lys-88, and Met-90 and that Met-90 is probably the major species-specific determinant.**

The scrapie agent is a unique pathogen in that an abnormal host protein is the only agent-specific macromolecule identified; an agent-specific nucleic acid has not been demonstrated. The human diseases kuru, Creutzfeldt-Jakob disease, and Gerstmann-Straussler syndrome are caused by related etiologic agents and show similar molecular pathology. A cattle disease analogous to scrapie, termed bovine spongiform encephalopathy, emerged recently in Great Britain, and epidemiologic studies indicate that the probable source of the epidemic was scrapie agent-contaminated protein feed supplement produced from rendered sheep carcasses (51, 55, 57). The human and animal diseases are characterized by prolonged asymptomatic incubation periods, followed by defined clinical phases and death. There is no detectable immune response to the etiologic agents, although the agents accumulate in the spleen and lymph nodes before the onset of clinical symptoms (1).

Current knowledge of the structure and composition of the scrapie agent precludes its classification within any known virus family (46). The only macromolecule that has been conclusively demonstrated to be specifically associated with these diseases, or the etiologic agents, is an abnormal host glycoprotein. This protein has been referred to as PrP, PrP-27-30, SAF protein, PrP 33-35$^{Sc}$, PrP$^{Sc}$, PrP$^{35-38}$, and Sp33-37 (5, 9, 13, 23, 30, 41, 45). (PrP-27-30 is produced from Sp33-37 by removal of the amino-terminal 67 residues when proteinase K is used during isolation [9, 10, 48]. PrP has been used to denote either protease-resistant protein or prion protein.) Sp33-37 accumulates in the brain and spleen during disease, with the maximum concentration being attained in the brain near the time of death. Sp33-37 has not been detected in normal brain at any time (6, 9, 12, 45), but the normal cellular form of this protein, Cp33-37, is synthesized in the brain throughout life beginning from the early neonatal period (42).

Sp33-37 is the major protein component in fractions containing the highly purified scrapie agent (9, 10, 48). There is abundant evidence that Sp33-37 plays an essential role in initiating the disease and promoting synthesis of the scrapie agent (6, 9, 10, 14, 17, 22, 23, 41, 50). Sp33-37 is resistant to protease degradation and aggregates into readily sedimentable forms during isolation. In vivo, at least some Sp33-37 is present in amyloid fibrils that accumulate in Congo red-staining, birefringent plaques (3, 49). These two properties, resistance to proteolytic degradation and aggregation, distinguish Sp33-37 from the normal cellular form, Cp33-37 (6, 45). The structural basis for the difference between Sp33-37 and Cp33-37 is not known, but it is likely that Sp33-37 is derived from Cp33-37 by a covalent posttranslational modification or a conformational change.

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Kascsak et al. (31) described a monoclonal antibody (MAb), designated 3F4, that was produced by immunizing mice with formic acid-denatured PrP-27-30 isolated from diseased hamster brains. MAb 3F4 binds to Sp33-37 and Cp33-37 from hamsters and humans, but it does not bind to either form of the protein from mice, rats, sheep, cattle, or rabbits (31). Thus, MAb 3F4 is a species-specific antibody, but it does not distinguish between the normal form (Cp33-37) and abnormal form (Sp33-37) of the protein. Comparison of the predicted amino acid sequences of PrP-27-30 from hamsters, humans, mice, and rats shows that differences within a single region of the protein correspond to the species specificity of MAb 3F4. In this region, which spans residues 87 to 90 in Syrian hamster scrapie agent isolate 263K Sp33-37 (Sp33-37$^{Ha}$), the Syrian hamster and human proteins have the sequence Met-Lys-His-Met, whereas mice have the sequence (Leu/Phe)-Lys-His-Val and rats have the sequence Leu-Lys-His-Val (33, 36, 37, 47, 56). Kascsak et al. suggested that the epitope for MAb 3F4 might be located in this region (31), but attempts to demonstrate binding of MAb 3F4 to synthetic peptides containing this sequence had been unsuccessful.

We immunized mice with formic acid-denatured, purified Sp33-37$^{Ha}$ in an attempt to produce MAbs to additional sites on the scrapie protein. In this report, we describe two new MAbs that were produced and demonstrate that these antibodies have the same species specificity and bind to the same site on Sp33-37$^{Ha}$ as does MAb 3F4. The molecular location of the epitope for these antibodies is identified to within 10 residues by using an antigen competition enzyme-linked immunosorbent assay (ELISA), and the chemical nature of the epitope was characterized by using a combination of chemical and enzymatic approaches.

## MATERIALS AND METHODS

**Production of MAbs.** Production of MAb 3F4 (immunoglobulin G2a [IgG2a]) was described by Kascsak et al. (31). MAb 4G8 was produced by using a synthetic peptide having the sequence of the Alzheimer's disease β/A4 β-amyloid peptide residues 1 to 24 (32). MAbs 4H8 and 6B11 were produced by immunizing female BALB/cJ mice (Jackson Laboratory, Bar Harbor, Maine) with monomeric Sp33-37$^{Ha}$, purified as described below. The mice were immunized via the back footpads with 200 μl of complete Freund's adjuvant containing 25 to 50 μg of Sp33-37$^{Ha}$ antigen, followed 21 days later by an intraperitoneal injection with 50 μg of Sp33-37$^{Ha}$ antigen in 200 μl of complete Freund's adjuvant. Four days prior to fusion, the mice were injected intraperitoneally with 200 μg of Sp33-37$^{Ha}$ without adjuvant. The spleen cells were fused with myeloma cells at a ratio of 5:1. Polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, Ind.) was used as the fusing agent.

A mouse with the highest antibody titer to Sp33-37$^{Ha}$ after immunization with the purified antigen was selected for hybrid clone production, and spleen cells were fused with the NSO strain of myeloma cells. Hybrid cell growth was detected in 14 of 300 wells after 2 to 4 weeks of incubation. Antibodies specific to Sp33-37$^{Ha}$ were detected in the supernatant fluid from 3 of 14 hybrids by the ELISA technique as described by Kim et al. (32). Hybrids that produced Sp33-37$^{Ha}$-specific antibodies were cloned three times by limiting dilutions to obtain pure clones, and two stable cloned hybrid cell lines from each were established. Ascites fluid was obtained by intraperitoneal injection of $10^7$ cells from cloned hybrid cell lines that had continued to produce antibody for 3 months. BALB/cJ mice were treated with 0.25 ml of pristane (2,6,10,14-tetramethylpentadecane) 8 days and 1 day before injection of the cells.

**Characterization of antibody subclasses.** The immunoglobulin subclasses of MAb 4H8 (IgG2b) and MAb 6B11 (IgG1) were determined by Ouchterlony double-diffusion tests with goat anti-mouse antibodies specific to IgG1, IgG2a, IgG2b, IgG3, and IgM (Meloy Laboratories Inc., Springfield, Va.). The concentration of antibodies in the ascites fluid was determined by single radial immunodiffusion assay using subclass-specific antibodies (Tago, Inc., Burlingame, Calif.).

**Purification of the scrapie agent and Sp33-37.** The 263K isolate of the scrapie agent was propagated and purified from the brains of diseased Syrian hamsters as described previously (9) with subsequent modifications (6). The $P_5$ fraction is highly enriched for both the scrapie agent (specific activities for hamster 263K scrapie agent are between $10^{10}$ and $10^{11}$ 50% lethal doses per mg of protein) and Sp33-37$^{Ha}$, which accounts for about 70 to 90% of the protein as detected by silver staining or protein sequencing (9). The ME7 mouse scrapie agent was propagated and purified from the brains of clinically affected C57BL/6J mice by the same procedure. Monomeric Sp33-37$^{Ha}$ and mouse ME7 Sp33-37 (Sp33-37$^{Mo}$) were separated from dimers and higher-order aggregates by size exclusion chromatography in 70% formic acid on a Superose 12 HR 10/30 column.

**Purification and characterization of synthetic peptides.** Peptides A111-78 and A111-79 were synthesized by Multiple Peptide Systems (San Diego, Calif.), and peptides FM-23S and FM-24S were synthesized by Biosearch Research Chemicals (San Rafael, Calif.). All four peptides were synthesized in the C-terminal amide form. The sequences and molecular locations of each peptide are shown in Table 2. Each peptide was purified by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C18 protein/peptide column (4.6 by 250 mm, 5 μm, 30 nm), using a dual-pump, high-pressure mixing system (Kratos/ABI model 150). The column was equilibrated at 45°C in 95% solvent A (0.1% trifluoroacetic acid)–5% solvent B (60% acetonitrile, 0.085% trifluoroacetic acid in water), and the peptides were eluted after a 1-min delay, using a linear gradient increasing at a rate of 3.75% solvent B per min at a flow rate of 600 μl/min. For each purified peptide, material from several chromatographic separations was pooled and stored as a stock solution. The stock solutions of each peptide were quantified by phenylthiocarbamyl amino acid analysis after hydrolysis in vacuo with 6 N HCl in the gas phase at 150°C for 1 h (7, 9, 27). The complete amino acid sequence of each purified peptide was determined by gas-liquid phase microsequencing, using an ABI model 470A updated with an on-line phenylthiohydantoin amino acid analyzer and data collection-analysis station (model 900/120A; Applied Biosystems Inc., Foster City, Calif.). The method used was essentially that described by Hewick et al. (28). The amino acid analysis and sequencing results verified the structure of each peptide.

**ELISAs. (i) Antibody competition ELISA.** Each MAb was purified from the ascites fluid by using the Affi-Gel Protein A MAPS system (Bio-Rad Laboratories, Richmond Calif.), and some of each purified MAb was conjugated to horseradish peroxidse (HRP) by the periodate method of Wilson and Nakane (58). The HRP-conjugated MAb solutions were stored at −70°C in small aliquots containing 1% bovine serum albumin (BSA), 50% glycerol, and 0.01% thimerosal. For each MAb, a fixed amount of HRP-conjugated antibody was mixed with 1, 10, or 100 μg of either the unlabeled

homologous MAb or one of the heterologous MAbs per ml. The mixtures were added to ELISA plate wells coated with Sp33-37$^{Ha}$ antigen at a concentration of 4 nM, and the plates were developed by standard ELISA methods.

**(ii) Direct ELISA.** Wells of a 96-well microtiter plate were coated with 50 μl of an appropriate antigen at the concentrations shown in Fig. 4. The antigens were diluted 100-fold from a concentrated stock in 70% formic acid into 0.12 M sodium carbonate buffer (initial pH 9.5). The final pH of the coating solution was less than 4. The plates were kept overnight at room temperature, washed with phosphate-buffered saline (PBS) and blocking buffer (0.2% BSA, 0.05% Tween 20 in PBS), and incubated for 1 h in blocking buffer. The plates were usually stored at −20°C after blocking. Primary antibody (50 μl) diluted in blocking buffer was added to each well. The plates were kept at room temperature for 1 h and then washed four times with blocking buffer. Then 200 μl of blocking buffer was added for 10 min, the wells were drained, and the plates were kept at room temperature for 1 h after the addition of 60 μl of secondary antibody. The secondary antibody, goat anti-mouse IgG plus IgM conjugated with alkaline phosphatase (Tago), was diluted in blocking buffer. Following four washes with blocking buffer and four washes with Tris-buffered saline (25 mM Tris [pH 7.4], 0.14 M NaCl, 2.7 mM KCl), 60 μl of PNPP in substrate buffer (as specified by the manufacturer [Sigma Chemical Co., St. Louis, Mo.]) was added and the plates were incubated at 37°C. $A_{405}$ was read 0, 15, 30, 45, and 60 min after addition of the substrate. Most of the ELISA manipulations were performed by a Biomek 1000 robot (Beckman Instruments, Fullerton, Calif.). The data were plotted and analyzed by using Sigmaplot (Jandel Scientific, Corte Madera, Calif.).

**(iii) Antigen competition ELISA.** The competing antigen was diluted in 70% formic acid, frozen in a dry ice-95% ethanol bath, and vacuum extracted to dryness. The dried antigens were dissolved in blocking buffer containing the diluted MAb of interest and incubated for 1 h at 37°C or room temperature. The resulting MAb-antigen solution was substituted for the primary antibody, and the direct ELISA procedure was followed with the exceptions noted below. In the competitive ELISA method, each well was coated with 117 fmol of HPLC-purified monomeric Sp33-37$^{Ha}$ diluted 100-fold in carbonate buffer from stock in 70% formic acid. The results are expressed as percent inhibition. The 0 and 100% inhibition standards consisted of primary antibody without competing antigen and buffer without primary antibody, respectively.

**SDS-PAGE and immunoblotting.** One-dimensional electrophoresis into sodium dodecyl sulfate (SDS)-polyacrylamide gels (SDS-PAGE) was performed essentially as described by Laemmli (34). Slab gels containing 15% total acrylamide were used for analysis of Sp33-37$^{Ha}$ and Sp33-37$^{Mo}$. The separated proteins were analyzed by staining with silver (8) or transferred to nitrocellulose for immunoblotting. Immunoblots were prepared by electrophoretic transfer of proteins to nitrocellulose sheets, using a Bio-Rad Trans Blot Cell apparatus (15, 53). The immunoblots were developed as described previously (6, 9, 35).

## RESULTS

**Species specificity of the MAbs.** MAbs 4H8 and 6B11 were tested by SDS-PAGE immunoblotting for binding to Sp33-37$^{Ha}$ and Sp33-37$^{Mo}$ (Fig. 1). As reported for MAb 3F4, MAbs 4H8 and 6B11 bound to hamster Sp33-37$^{Ha}$ but did not bind to Sp33-37$^{Mo}$, even though eight times more protein was loaded per lane. These results were confirmed by direct ELISA.

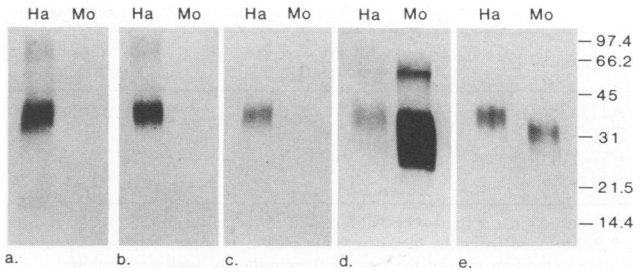


FIG. 1. Binding of 4H8 and 6B11 to Sp33-37$^{Ha}$ but not to Sp33-37$^{Mo}$. Polyacrylamide gels were loaded with 3.8 ng (150 fmol) of HPLC-purified Sp33-37$^{Ha}$ (Ha) or 30 ng (1.2 pmol) of HPLC-purified Sp33-37$^{Mo}$ (Mo) per lane. After transfer to nitrocellulose, the blots were incubated with 3F4 (1:50,000; a), 4H8 (1:50,000; b), or 6B11 (1:20,000; c) and developed by using the alkaline phosphatase-conjugated goat anti-mouse secondary antibody/5-bromo-4-chloro-3-indolyl phosphate/nitrotetazolium blue system. Another gel was loaded with 38 ng (1.50 pmol) of HPLC-purified Sp33-37$^{Ha}$ (Ha) or 300 ng (12 pmol) of HPLC-purified Sp33-37$^{Mo}$ (Mo) per lane, and the immunoblot was incubated with a polyclonal antibody that binds both Sp33-37$^{Ha}$ and Sp33-37$^{Mo}$ (3, 4) and developed by using goat anti-rabbit secondary antibody (d). The purity of the Sp33-37 preparations is demonstrated in the silver-stained gels (e). Molecular sizes (in kilodaltons) are shown on the right.

**MAbs 3F4, 4H8, and 6B11 bind to the same site.** The observation that MAbs 3F4, 4H8, and 6B11 shared species specificity suggested that they might bind to the same epitope. We used an antibody competition ELISA to test this hypothesis (Table 1). Each MAb was purified by protein A affinity chromatography and conjugated directly with HRP. Binding of each HRP-conjugated MAb to HPLC-purified Sp33-37$^{Ha}$ was tested in the presence of each of the other MAbs as well as MAb 4G8, an MAb to the Alzheimer's disease β/A4 peptide. In each case, inclusion of 3F4, 4H8, or 6B11 inhibited binding of each of the HRP-conjugated antibodies (Table 1). None of the antibodies was inhibited by MAb 4G8. The results demonstrated that 3F4, 4H8, and 6B11 bind to the same site or overlapping sites on Sp33-37$^{Ha}$.

**Molecular location of the antibody binding site.** Other studies suggested that the binding site for MAb 3F4 is a linear sequence-specific epitope that includes Lys-88 and Met-90 (31; see below). We attempted to measure direct binding of MAb 3F4 to two hamster-specific synthetic peptides encompassing this region (Table 2). Control peptides

TABLE 1. Competition by MAbs 3F4, 4H8, and 6B11 for the same binding site

| HRP-conjugated Mab | Dilution[a] | Normalized % inhibition[b] with given blocking MAb (100 μg/ml) | | |
|---|---|---|---|---|
| | | 4H8 | 6B11 | 4G8 |
| 3F4 | 1:200 | 86 | 61 | 0 |
| 4H8 | 1:200 | 86 | 71 | 3 |
| 6B11 | 1:50 | 99 | 68 | 1 |

[a] Determined by ELISA, using plates coated with Sp33-37$^{Ha}$ at approximately 50 ng per well.

[b] Calculated from the relative binding of each HRP-conjugated MAb in the presence or absence of the competing antibody. Values were normalized to those for 3F4 as the competing antibody (set at 100%).

TABLE 2. Synthetic peptides

| Designation | Length (amino acids) | Sequence[a] | Species | Positions[b] |
|---|---|---|---|---|
| A111-78 | 15 | K P S K P K T N M K H M A G A | Hamster | 79–93 |
| FM-24S | 10 | K T N M K H M A G A | Hamster | 84–93 |
| FM-23S | 10 | K T N L* K H V* A G A | Mouse | 83–92 |
| A111-79 | 14 | M L G S A M S R P M M H F G | Hamster | 107–120 |

[a] Asterisks mark the two positions that differ between the mouse and hamster sequences in this region.
[b] Referenced from the signal peptide cleavage point, with the signal peptide extending toward the amino terminus as negative numbers and the processed protein extending toward the carboxyl terminus as positive numbers:

$$M_{-22}ANL.....DVGLC_{-1}\downarrow\uparrow K_1KRPKP....FLMVG_{232}$$

included a peptide with the mouse-specific sequence from this region and another peptide with the sequence from a different region of Sp33-37$^{Ha}$. MAb 3F4 did not specifically bind to the peptides in slot blots (A111-78) or in ELISAs (A111-78 or FM-24S), even when the peptides were used at molar concentrations up to 100-fold higher than the concentration of Sp33-37$^{Ha}$ (data not shown).

We reasoned that attachment of the relatively short peptides to the solid substrates used in these assays might prevent the antibody from binding. Therefore, an antigen competition ELISA was developed to determine whether any of the peptides would specifically bind to the antibodies in solution. In this technique, the peptide and the antibody were mixed in solution and incubated for 1 h to allow binding. The antigen-antibody solution was then transferred to an ELISA plate precoated with purified Sp33-37$^{Ha}$. After washing, the antibody attached to Sp33-37$^{Ha}$ (a measure of the noncompeted antibody) was detected by standard ELISA methods. Binding of a test peptide (the competing soluble antigen) to an antibody molecule prevented the antibody from binding to the solid-phase antigen and thus gave a negative ELISA result. The results were expressed as percent inhibition.

Sp33-37$^{Ha}$, Sp33-37$^{Mo}$, and the four synthetic peptides were used as competing antigens over a range of concentrations from 30 pM to 30 μM in a competition ELISA with MAb 3F4 (Fig. 2). Purified monomeric Sp33-37$^{Ha}$ was an effective competing antigen at a concentration of 300 pM, gave 50% inhibition at 3 nM, and gave essentially complete inhibition at 30 nM. Synthetic peptides A111-78 and FM-24S were effective competing antigens over the same range of concentrations (3 to ≥300 nM), demonstrating that these peptides bind specifically to MAb 3F4. The relative affinities of these antigens for MAb 3F4 were A111-78 > Sp33-37$^{Ha}$ > FM-24S. FM-23S, which differs from FM-24S by only two amino acids (Table 2), did not bind to 3F4 at concentrations of ≤3 μM and bound only slightly at 30 μM. FM-23S at 30 μM inhibited to a similar extent as 3 nM FM-24S, a concentration difference of 10,000-fold. Purified Sp33-37$^{Mo}$ and synthetic peptide A111-79 did not bind measurably to 3F4 over the range of concentrations tested (Fig. 2).

We examined the inhibition of antibody binding by synthetic peptides in more detail by using twofold dilutions of the competing antigens over a narrow concentration range. MAb 4H8 gave results (Fig. 3) similar to those obtained with MAb 3F4 over the broader range of antigen concentrations (Fig. 2). As before, the relative affinities of the antigens were A111-78 > Sp33-37$^{Ha}$ > FM-24S. Similar results were obtained for MAb 6B11 at a dilution of 1:40,000 (data not shown).

The concentration of antibody molecules in the ascites fluid of each MAb was measured by using radial immunodiffusion with subclass-specific antibodies: 3F4 (120 μM), 4H8 (21 μM), and 6B11 (21 μM). These data, combined with the antigen competition ELISA results, indicate that these antibodies have dissociation constants in the nanomolar range. The relative order of the affinities for the antigens was MAb 4H8 > 3F4 > 6B11.

**MAbs show reduced binding to peptides by direct ELISA.** The fact that MAbs 3F4, 4H8, and 6B11 specifically bound the hamster synthetic peptides in solution suggested that the binding of MAb 3F4 to the synthetic peptides coated on polystyrene ELISA plates should be reexamined. ELISA plates were coated with Sp33-37$^{Ha}$, Sp33-37$^{Mo}$, A111-78, FM-24S, and FM-23S at selected concentrations between 30 pM (1.5 fmol per well) and 30 μM (1.5 nmol per well). In accord with our previous experience, plates coated with Sp33-37$^{Ha}$ at 300 pM produced significant absorbance, and plates coated at a concentration of 3 nM (150 fmol per well) gave absorbance values greater than 2 when measured at 60 min (Fig. 4). By contrast, peptide A111-78 produced significant absorbance (0.66 ± 0.14) only at a concentration of 300

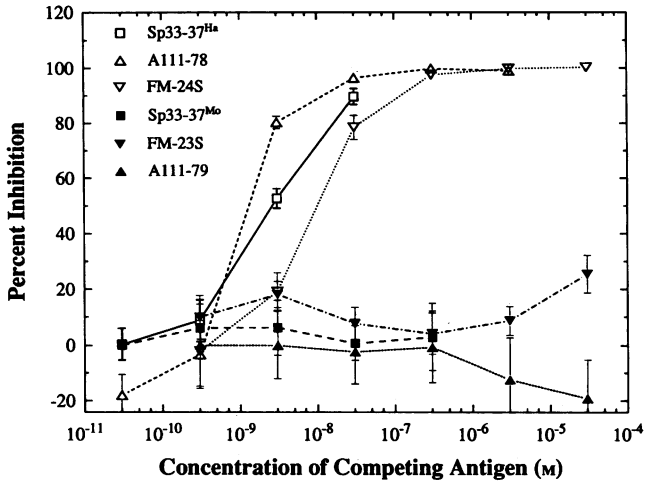


FIG. 2. Specific binding of synthetic peptides of MAb 3F4 in an antigen competition ELISA. Dilutions of Sp33-37$^{Ha}$, Sp33-37$^{Mo}$, A111-78, FM-24S, FM-23S, and A111-79 were dried in microcentrifuge tubes and dissolved in blocking buffer containing MAb 3F4 diluted 1:200,000. The antigen-MAb 3F4 solutions were incubated for 1 h at 37°C, and then free MAb 3F4 was measured by the competition ELISA method. Absorbance values at 60 min were converted to percent inhibition. The error bars show the standard deviation.

TABLE 3. Predicted sequences of hamster, human, mouse, rat, and sheep Cp33-37/Sp33-37 and MAb 3F4 binding

| Species | Positions | Sequence[a] | MAb 3F4 binding | Reference(s) |
|---|---|---|---|---|
| Syrian hamster | 79–93 | K P S K P K T N M K H M A G A | + | 47 |
| Armenian hamster | 79–93 | . . N . . . . S . . . . . . . | + | 38 |
| Chinese hamster | 79–93 | . . . . . . . . . . . . V . . . | − | 38 |
| Human | 79–93 | . . . . . . . . . . . . . . . | + | 33 |
| Mouse (RML, NZW)[b] | 78–92 | . . . . . . . . . L . . V . . . | − | 37, 56 |
| Mouse (ILn/J)[c] | 78–92 | . . . . . . . . . F . . V . . . | − | 56 |
| Rat | 79–93 | . . . . . . . . . L . . V . . . | − | 36 |
| Sheep | 80–94 | . . . . . . . . . . . . V . . . | − | 24 |

[a] Positions with sequence identity to the hamster sequence are shown with a dot. The sequences are numbered according to the convention designating the signal peptide as residues −22 to −1 and the processed protein as residues 1 to 232, based on the sequence of the Syrian hamster protein.
[b] Have the short incubation phenotype with ME7 or similar scrapie agent isolates (56).
[c] Has the long incubation phenotype with ME7 scrapie agent (56).

nM, and peptide FM-24S gave an absorbance reading of 0.33 ± 0.09 at a concentration of 30 μM.

The coating solution used in those studies was 0.7% formic acid–0.12 M sodium carbonate (pH < 4) because of the need to dilute the purified protein samples from 70% formic acid stock solutions. It was possible that under those conditions the peptides bound inefficiently to the polystyrene ELISA plates, so we tested a coating solution with a higher pH value. When coated at pH 9.45, 300 nM and 3 μM concentrations of A111-78 gave significantly higher absorbance values than did the same concentrations coated in the low-pH buffer, but the wells coated with A111-78 at concentrations of ≤30 nM did not bind antibody regardless of the pH of the coating buffer (data not shown).

**Chemical modification of the epitope.** Diethylpyrocarbonate (DEP) reversibly inactivates the scrapie agent, probably by modification of one or more His residues (43). Under the same reaction conditions, PrP-27-30 (a fragment of Sp33-37 produced by partial digestion with proteinase K) is labeled with $^{14}$C-DEP (10, 11), but the sites on Sp33-37 or PrP-27-30 that are modified by DEP have not been defined. Preliminary studies were conducted to identify residues modified by DEP that might be responsible for inactivation of the scrapie agent. Analysis of the modified protein by SDS-PAGE immunoblotting showed that DEP modification of Sp33-37 reduced binding of monoclonal antibody 3F4 in a dose-dependent manner. Significant effects were observed at DEP concentrations as low as 1 mM, and binding was eliminated after modification at concentrations above 5 mM (data not shown). MAb 3F4 binding was not restored by hydroxylamine (data not shown), in contrast to the reversibility of DEP inactivation of the scrapie agent (43; data not shown). In comparison with MAb 3F4, the binding of a polyclonal serum to Sp33-37 was less affected by DEP modification.

Irreversible modification by DEP is characteristic of ethoxyformylation of Lys rather than His (44). Therefore, Sp33-37 was modified with succinic anhydride to determine whether one or more Lys residues are essential for 3F4 binding. Succinylation of Sp33-37 under nondenaturing conditions did not diminish MAb 3F4 binding, but addition of SDS to the succinylation reaction buffer enhanced modification of residues within the MAb 3F4 epitope and dramatically reduced binding of MAb 3F4 to the protein (Fig. 5).

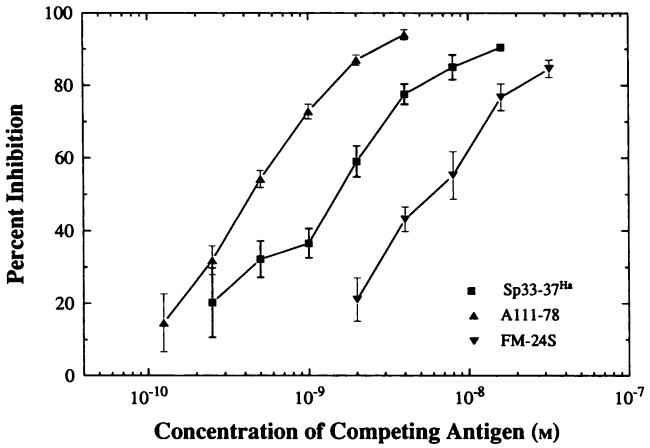


FIG. 3. Dose response of competing antigens with MAb 4H8. Twofold dilutions of Sp33-37$^{Ha}$, A111-78, and FM-24S were dried in microcentrifuge tubes and dissolved in blocking buffer containing MAb 4H8 diluted 1:200,000. The antigen-MAb 4H8 solutions were incubated for 1 h at room temperature, and then free MAb 3F4 was measured by the competition ELISA method. Absorbance values at 60 min were converted to percent inhibition. The error bars show the standard deviation.

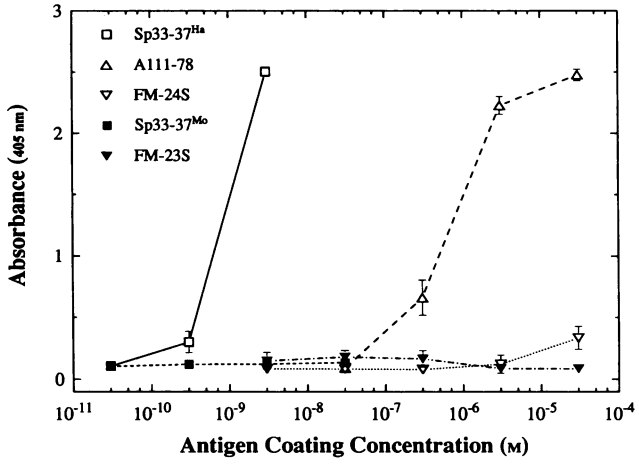


FIG. 4. Reduced binding of synthetic peptides in direct ELISA. Polystyrene plates were coated (50 μl per well) with Sp33-37$^{Ha}$, Sp33-37$^{Mo}$, A111-78, FM-24S, and FM-23S at the concentrations indicated. The plates were washed, blocked, and developed with MAb 3F4 diluted 1:200,000. Sixty-minute absorbance values are shown. The error bars show the standard deviation. The standard coating concentration used in the competition ELISAs shown in Fig. 2 and 3 was 117 fmol per well, equivalent to a coating concentration of 2.3 nM.

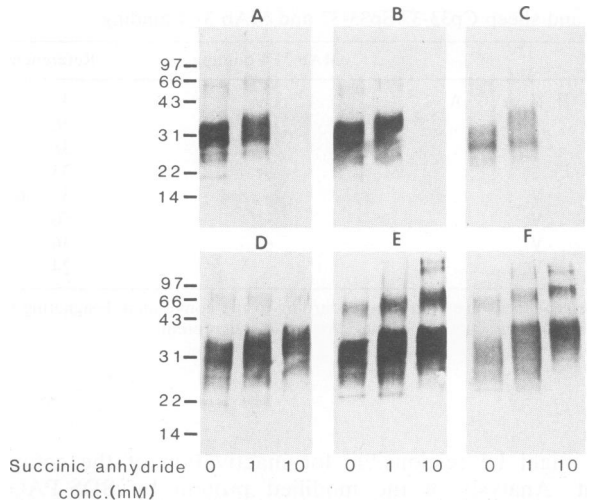


FIG. 5. Modification with succinic anhydride and loss of MAb 3F4 binding. Sp33-37 in purified scrapie agent fraction $P_5$ was incubated for 5 min at 22°C (A, B, D, and E) or 100°C (C and F) in 1% $NaCO_3$ buffer (pH 8.0) containing 0.3% (A and D) or 2% (B, C, E, and F) SDS. The protein was succinylated by addition of 1/10 volume of succinic anhydride, in absolute ethanol, in two aliquots at 0 and 30 min. The mixture was allowed to stir for a total of 60 min. (A to C) Immunoreactivity with the 3F4 antibody; (D to F) Immunoreaction with the anti-PrP-27-30 polyclonal serum.

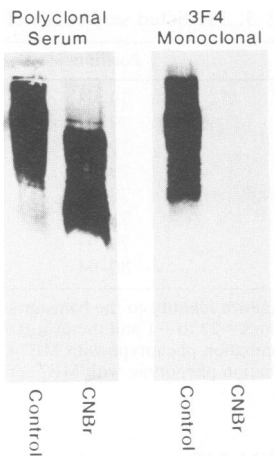


FIG. 6. CNBr cleavage of Sp33-37. A sample of the scrapie agent $P_5$ fraction was diluted with 4 volumes of methanol and sedimented at 16,000 × *g* for 30 min at 4°C. The pellet was suspended in 70% formic acid. One half of the suspension was treated with CNBr (50 mg/ml, final concentration), and the other half was treated with 70% formic acid alone for 5 h. PAGE and immunoblotting revealed cleavage of the protein with use of the polyclonal serum. The MAb 3F4 epitope was lost during this treatment.

This effect was seen even at low concentrations of SDS after incubation at 22°C (Fig. 5A). Immunostaining of the succinylated protein by the polyclonal serum demonstrated that other epitopes were not significantly affected by succinylation under these conditions (Fig. 5D to F). Sp33-37 contains 11 Lys residues, with clusters of three and four Lys residues, respectively, in the regions spanning positions 1 to 5 and 79 to 88. The other four Lys residues are spread across the region spanning residues 163 to 198.

Contrary to the results obtained with DEP and succinic anhydride, we found that MAb 3F4 binding was only slightly reduced by modification of Sp33-37 with *N*-succinimidyl 3-(4-hydroxyphenyl) propionate (Bolton-Hunter reagent) in the presence of 0.1% SDS (data not shown). Also, MAb 3F4 binding was not diminished after chloramine-T iodination in buffer containing 0.1% SDS (data not shown). While these reagents have previously been shown to modify PrP-27-30 (10, 11, 13), the modified residues have not been identified. The Bolton-Hunter reagent, like succinic anhydride, is relatively specific for Lys residues; chloramine-T iodination can modify His and Cys residues in addition to Tyr.

**Chemical fragmentation.** Chemical fragmentation with CNBr was used to further define the essential residues within the MAb 3F4 epitope. We found that MAb 3F4 failed to bind to any peptide fragment following CNBr cleavage (Fig. 6), consistent with participation of Met-87 or Met-90 as a determinant of this epitope. Incubation in formic acid alone had no effect on MAb 3F4 binding, demonstrating that cleavage at a methionine residue, and not formic acid denaturation, destroyed the MAb 3F4 epitope. Several peptides were identified by a polyclonal serum, indicating that other epitopes were preserved after cleavage (Fig. 6).

**Enzymatic digestion.** MAb 3F4 binding was virtually eliminated after trypsin digestion of denatured Sp33-37 but was preserved after digestion with pepsin (not shown). Pepsin digestion produced two peptides that retained the 3F4 epitope after isolation by size exclusion HPLC in 70% formic acid and by reverse-phase HPLC. The sequence of the isolated peptides was not determined because the amino terminus was found to be blocked.

## DISCUSSION

This study shows that three MAbs to $Sp33\text{-}37^{Ha}$ bind specifically to synthetic peptide FM-24S and thus places the epitope for MAbs 3F4, 4H8, and 6B11 in the region encompassing residues 84 to 93 of $Sp33\text{-}37^{Ha}$. By contrast, a peptide with the corresponding mouse sequence was essentially not bound by these antibodies, its affinity being approximately 10,000-fold lower. The fact that these two peptides differ by only two conservative amino acid substitutions ($Met_{Ha}$-87 → $Leu_{Mo}$-86 and $Met_{Ha}$-90 → $Val_{Mo}$-89) demonstrates the molecular specificity of the antibodies and explains their species-specific immunoreactivity.

Production of three distinct MAbs to a single site in $Sp33\text{-}37^{Ha}$ suggests that this region is an immunodominant epitope, as seen in the mouse immune system. Within the sequence of Sp33-37, the Syrian hamster and *Prn-p*$^a$ genotype mouse proteins are 95% identical at the amino acid sequence level (2, 9, 16, 52, 56). There are 11 differences in 209 residues, but none of these occur at charged residues and only two would be considered nonconservative. One of these differences arises from the substitution of a nonpolar for a polar residue ($Thr_{Ha}$-193 → $Val_{Mo}$-192), and the other results from a deletion in the mouse protein ($Gly_{Ha}$-33 → $\Delta_{Mo}$). The other sequence differences are four conservative substitutions of polar residues and five conservative substitutions of nonpolar residues. In view of the degree of sequence identity between the hamster and mouse proteins, it is not surprising that MAbs have been produced to only a limited number of sites.

Table 3 compares binding of MAb 3F4 with the protein sequences of the region containing the 3F4/4H8/6B11 epitope from several different mammalian species. Binding of the 3F4 antibody correlates with the presence of methio-

nine at residue 90 in the protein from Syrian hamsters, Armenian hamsters, and humans. Substitution of Val for Met at position 90 in the Chinese hamster and sheep proteins is apparently sufficient to significantly reduce or eliminate MAb 3F4 binding (24, 38). It is not known whether a single substitution for Met-87 would eliminate binding by the antibodies. Asn-86 apparently is not essential because MAb 3F4 binds to the Armenian hamster protein which has Ser at this position (38). It is not known to what extent this substitution affects the affinity of MAb 3F4 for the protein, however.

It is evident from the foregoing discussion that Met-90 is essential for 3F4 binding. A reasonable approximation for the average size of a linear protein antigenic determinant is five to seven amino acids (29). If we assume that the 3F4/4H8/6B11 epitope includes seven amino acids and that the carboxy terminus is at Met-90, the amino terminus of the epitope would be Lys-84. Loss of MAb 3F4 binding after modification with succinic anhydride demonstrates that at least one Lys residue is essential. Thus, Lys-84, Lys-88, or both could be required for high-affinity binding of these MAbs.

It could not be determined from our experiments whether modification of His-89 by DEP correlated with loss of scrapie agent activity. Treatment with hydroxylamine restored scrapie agent activity, but it failed to restore binding of MAb 3F4 to DEP-modified Sp33-37. Thus, the effect of DEP on 3F4 binding could have resulted from modification of Lys-84 or Lys-88 regardless of any modification of His-89. Other studies will be needed to determine whether His-89 is essential for scrapie agent activity. Chloramine-T iodination of Sp33-37 did not measurably affect MAb 3F4 binding, consistent with the absence of Tyr residues within the epitope, but this method can also produce mono- and diiodinated His (44). If His-89 was iodinated in our experiments, its iodination did not affect MAb 3F4 binding. Thus, iodination of Sp33-37 by this method could provide a useful means for chemically labeling the intact protein or Tyr-containing peptides while retaining MAb 3F4 immunoreactivity.

Data from several experimental approaches confirm that the 3F4/4H8/6B11 epitope is a linear, sequence-specific site that contains no posttranslational modifications. Denaturation of Sp33-37 or PrP-27-30, by boiling in SDS (Fig. 1, 5, and 6; 31) or prolonged incubation in 70% formic acid (Fig. 6), did not diminish MAb 3F4 binding. Pepsin digestion of Sp33-37 produced peptides of ~10 kDa that retained the MAb 3F4 binding site after denaturation in formic acid and separation by SDS-PAGE (data not shown). MAb 3F4 bound to picomole amounts of the denatured protein electroblotted onto nitrocellulose paper or adsorbed to polystyrene ELISA plates (Fig. 1 to 4; 31). Finally, 10- and 15-residue synthetic peptides bound to 3F4 in solution with affinities approximately equal to or greater than that of the intact protein (Fig. 2 and 3).

Our studies provide some information regarding the conformation of the protein near the MAb 3F4 epitope. Incubation of nondenatured Sp33-37 with succinic anhydride at concentrations of up to 10 mM produced increasing modification of the protein but did not affect the Lys residues within the MAb 3F4 epitope. Addition of low concentrations of SDS apparently unfolded the protein enough to promote succinylation of one or more Lys residues in the epitope and eliminate MAb 3F4 binding. These data suggest that the Lys residues within the MAb 3F4 epitope are less accessible to the solvent than are other Lys residues and that some degree of unfolding of the protein may be required for modification by succinic anhydride. The fact that *N*-succinimidyl 3-(4-hydroxyphenyl) propionate also modified Lys residues in Sp33-37, but apparently not those within the MAb 3F4 epitope, is consistent with this hypothesis. It may be significant that this site lies immediately to the amino-terminal side of a 20-amino-acid hydrophobic region (47).

We consistently observed that synthetic peptide A111-78 had a higher affinity than peptide FM-24S for the three MAbs. A111-78 is five residues longer, and thus amino acids upstream of the amino terminus of FM-24S, e.g., Pro-83 and Lys-82, may increase binding affinity by interacting directly with the antibody binding site. Alternatively, the longer peptide may enhance binding indirectly by giving A111-78 a more favorable conformation within residues 84 to 93. It is not clear why peptide A111-78 is bound more efficiently than Sp33-37$^{Ha}$, but it may be that some conformations of the denatured protein hinder antibody binding. Sp33-37$^{Ha}$ has a strong tendency to aggregate, and it is possible that some of the protein is aggregated in solution.

While the binding curves of Sp33-37$^{Ha}$, A111-78, and FM-24S with the MAbs in solution were very similar, they differed markedly when the antigens were coated directly on the ELISA plates. It is unlikely that the epitope per se is affected by binding to the solid phase since the protein antigen (Sp33-37$^{Ha}$) behaved the same in solution as when bound to the ELISA plate. It seems that peptide length is a significant factor affecting the presentation of the epitope when these peptides are adsorbed to the solid phase. Adsorption of peptides to a solid phase requires interaction between a portion of the peptide surface and the solid phase and therefore could preclude antibody binding if the contact surface overlaps the epitope. With peptide FM-24S, the length of the peptide may be insufficient to permit the peptide to bind to the solid phase and the antibody. The additional five residues present on A111-78 appear to enable some of the peptide to bind to the solid phase in a conformation that leaves most or all of the epitope free to bind antibody. The fact that the minimum effective coating concentration of A111-78 is about 1,000 times higher on a molar basis than that for Sp33-37$^{Ha}$ suggests that only a small percentage of the peptide binds to the solid phase in the proper conformation.

Using the 3F4 antibody, immunohistochemical localization of Sp33-37 or Cp33-37 has proved difficult even in tissues that contain substantial amounts (2a). Treatment of the tissues with formic acid prior to staining has been shown to improve detection with use of MAb 3F4 (unpublished data), consistent with our data suggesting that the epitope may be somewhat shielded from the solvent prior to denaturation. In some tissues in which the presence of the mRNA and protein has been documented biochemically, staining of the protein is not readily observed even after incubation in formic acid (2a). In this case, failure to stain the protein is unlikely to result from denaturation of Sp33-37 since MAb 3F4 binding is not decreased by denaturation. It could result either from modification of the binding site during fixation (e.g., modification of Lys with glutaraldehyde, formaldehyde, or paraformaldehyde) or from extraction of the protein from the tissue during dehydration or washing steps. In addition, Sp33-37 is readily soluble in formic acid, and so prolonged exposure to formic acid could extract the protein from the tissue (20). Therefore, when one is processing tissues for immunostaining with MAb 3F4, precautions should be taken to avoid fixatives, like glutaraldehyde, that

modify Lys residues and extensive extraction with organic solvents and formic acid.

Identification of the molecular location of the 3F4/4H8/6B11 epitope increases the utility of these MAbs as reagents for probing possible functions of Cp33-37. Cp33-37 is present on lymphocytes and may play a role as a lymphocyte activation molecule (19). Another function for Cp33-37 is suggested by sequence homology with the protein that purifies with the nicotinic acetylcholine receptor inducer activity protein isolated from chicken brain (25, 26, 54). It is not clear at present whether this protein is the chicken homolog of Cp33-37 or a closely related protein.

The antigen competition ELISA method and the MAbs described in this report are useful reagents for laboratory studies of hamster Sp33-37 and Cp33-37 but might also be used as a diagnostic tool to quantify the human Creutzfeldt-Jakob disease agent protein. High-affinity MAbs and a synthetic antigen could provide the basis for an effective diagnostic test of this type. We have found that peptide A111-78 can be used in place of purified $Sp33\text{-}37^{Ha}$ as the coating antigen in the antigen competition ELISA described in this report (50a). Creutzfeldt-Jakob disease has been transmitted to laboratory animals from human blood (40), and studies in animal models have shown that the agent is present in blood prior to onset of clinical disease (18, 21, 39). These observations suggest that a preclinical diagnostic blood test based on detection of Sp33-37 by ELISA might be possible. The test would have to distinguish between the normal and disease-associated forms of the protein, or reliably detect a significant increase in the concentration of the total pool (including both forms) in diseased versus normal individuals, if such differences exist. A similar course could be taken to develop a diagnostic test for bovine spongiform encephalopathy by using reagents specific for bovine Sp33-37 and Cp33-37.

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